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Calcium channel blockers, either amlodipine or mibefradil, ameliorate renal injury in experimental diabetes

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Calcium channel blockers, either amlodipine or mibefradil, ameliorate renal injury in experimental diabetes.

Background. Diabetic nephropathy is associated with increased albuminuria and accumulation of extracellular matrix proteins within the kidney. Clinical studies have shown some beneficial effects of calcium channel blockers (CCB) on diabetic nephropathy, even though they are generally considered to be less renoprotective than agents that interrupt the renin angiotensin system. However, effects of CCBs on renal injury, and in particular, expression of extracellular matrix proteins in a model of normotensive diabetic nephropathy, are poorly characterized.

Methods. Experimental diabetes was induced by injection of streptozocin in Sprague-Dawley rats. Amlodipine, a CCB which blocks the L channel, and mibefradil, a CCB blocking the T as well as the L channels, were given to diabetic rats for six months. Albumin excretion rate (AER), pathologic injury, and expression of the extracellular matrix proteins, collagen I, and fibronectin were assessed.

Results. Increased AER in diabetic rats (13.2 ×/÷1.3 mg/d, geometric mean ×/÷ tolerance factor) was attenuated by either amlodipine (3.2 ×/÷ 1.4 mg/d) or mibefradil (2.6 ×/÷ 1.4 mg/d). Increased glomerulosclerosis and tubulointerstitial injury in diabetic animals were attenuated by amlodipine and mibefradil. There was increased collagen accumulation in the kidney of diabetic rats as assessed by picro-sirius red staining. Gene expression of both collagen I and fibronectin were also increased in the kidneys from diabetic animals, as assessed by reverse transcription-polymerase chain reaction (RT-PCR). These markers of fibrosis were attenuated by treatment with either amlodipine or mibefradil. Blood pressure in diabetic rats (136 ± 2 mm Hg) was modestly reduced by amlodipine (126 ± 3 mm Hg) but not by mibefradil treatment (134 ± 3 mm Hg).

Conclusion. Calcium channel blockers attenuated albuminuria, pathologic injury, and accumulation of extracellular matrix proteins in this normotensive model of diabetic nephropathy. These findings suggest that CCBs may be useful in preventing pathologic injury in the diabetic kidney.

Key words: diabetic nephropathy, amlodipine, mibefradil, albuminuria, collagen I, fibronectin.

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Diabetic nephropathy is a leading cause of the development of end-stage renal disease (ESRD) and is a major cause of morbidity and mortality in the western world. One of the characteristic features of diabetic nephropathy is persistent albuminuria, particularly at the stage prior to ESRD [1, 2]. Experimental and clinical studies have suggested that albuminuria alone may be an independent risk factor for the development of diabetic nephropathy. Amelioration of albuminuria is one of the targets in preventing and retarding the progression of diabetic nephropathy [3].

Calcium channel blockers (CCBs) are widely used in the treatment of hypertension and in other forms of cardiovascular disease, such as stroke. Diabetic patients often have hypertension and cardiovascular conditions in which CCBs are indicated, and therefore, it is important to also have adequate information on their renoprotective role in this setting. Clinical studies have shown some beneficial effects of CCBs on diabetic nephropathy [1, 4], even though these agents are in general considered to be less renoprotective than agents that interrupt the renin angiotensin system (RAS), such as the angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor antagonists (ARB) [5]. Indeed, in normotensive diabetic patients, both ACE inhibitors and ARBs have been shown to be more effective in reducing albuminuria than CCBs [6, 7].

Hallmarks of pathologic injury in diabetic nephropathy include glomerulosclerosis and tubulointerstitial fibrosis [8]. Experimental diabetes in the rat is associated with accumulation of extracellular matrix proteins, including collagens and fibronectin, and this phenomenon is considered to be pivotal in the development of the overt manifestation of diabetic nephropathy [9]. However, effects of CCBs on renal injury, particularly on extracellular matrix protein expression in the model of normotensive diabetic nephropathy, are poorly characterized.

Calcium antagonists are a heterogeneous group of drugs, each with a different chemical structure and pharmacologic profile. There are several types of calcium channels, including the L channel and the T channel. Studies have shown that dihydropyridine CCBs such as

amlodipine blocks the L channel, whereas a newer calcium antagonist, mibefradil, has a unique chemical structure and blocks the T channel, as well as the L channel [10]. In the present study, we assessed the effects of both CCBs, amlodipine and mibefradil, on albuminuria, pathologic injury, and renal expression of the extracellular matrix proteins collagen I and fibronectin in experimental diabetes.

METHODS

Animals

Eight-week-old male Sprague-Dawley rats (body weight 230 to 280 g) were used in this study. The animal ethics committee of our institute approved the research protocol. Diabetes was induced by tail vein injection of strepozotocin (Boehringer-Mannheim, Mannheim, Germany) at a dose of 55 mg/kg in citrate following 16-hour fasting. Long-acting insulin (Ultralente, Novo Industries A/S, Copenhagen, Denmark) at a dose of 4 U/day was given to all diabetic animals by subcutaneous injection to avoid ketonuria and to maintain well-being. The animals had access ad libitum to water and standard rat chow (Clark King & Co., Melbourne, Australia).

Drug therapy

Following the induction of diabetes, the animals were randomly allocated into three groups and treated for 24 weeks: (*1*) diabetic rats with no treatment ($N = 10$); (*2*) diabetic rats treated with the dihydropyridine calcium channel blocker amlodipine (Pfizer, New York, NY, USA) at a dose of 20 mg per kg body weight per day in food admix (D + Amlodipine, $N = 11$); (*3*) diabetic rats treated with the non-dihydropyridine calcium channel blocker mibefradil (Roche, Basel, Switzerland) at a dose of 20 mg per kg body weight per day in food admix (D + Mibefradil, $N = 12$). In addition, nondiabetic rats were used as control animals ($N = 10$).

Systolic blood pressure (SBP) was measured by tail-cuff plethysmography in conscious, preheated rats [11] every four weeks, and body weight was measured. In brief, rats were restrained and warmed with a heat lamp for five minutes before measurement, then wrapped in a towel with the tail exposed, allowing access to the tail-cuff. An occlusive plethysmograph was attached to a pneumatic pulse transducer (Narco Bio-system, Inc., Houston, TX, USA). Three measurements were taken over five minutes and the mean value calculated.

Animals were placed in metabolic cages at week 24 (Iffa Credo, L'Arbresele, France) for collection of urine over 24 hours for measurement of albumin concentration by radioimmunoassay as previously described [12]. Blood samples were collected from the tail veins of conscious rats before the animals were sacrificed for measurement of glycated hemoglobin (HbA1c). HbA1c was measured by a high-performance liquid chromatography method (Bio-Rad, Richmond, CA, USA) [13].

Kidney collection

At the conclusion of the experiment, animals were anesthetized by intravenous injection of pentobarbitone sodium (60 mg/kg body weight, Boehringer Ingelheim, Artarmon, NSW, Australia). A midline incision of the abdomen was cut and the right kidney was removed and weighed. The kidney was bisected and fixed in 10% formalin, and processed to paraffin for subsequent histologic assessment, in situ hybridization, and immunohistochemical studies.

Kidney histopathology

Glomerulosclerosis and tubulointerstitial injury were performed using semiquantitative scores as described previously [14]. Kidney sections were stained with hematoxylin and eosin and observed under a light microscope in a masked fashion at a magnification of ×400 using the Imaging Analysis System (AIS, Imaging Research, St. Catherines, Ontario, Canada) associated with a video camera and computer. All glomeruli in each kidney section were graded according to the severity of the glomerular damage: 0, normal; 1, slight glomerular damage, the mesangial matrix and/or hyalinosis with focal adhesion, involving <25% of the glomerulus; 2, sclerosis of 25% to 50%; 3, sclerosis of 50% to 75%; 4, sclerosis of >75% of the glomerulus. The tubulointerstitial area in the cortex was observed and graded as: 0, normal; 1, the area of interstitial inflammation and fibrosis, tubular atrophy, and dilation with cast formation involving <25% of the field; 2, lesion area between 25% and 50% of the field; and 3, lesions involving >50% of the field. The indices for glomerulosclerosis or tubulointerstitial injury were calculated by averaging the grades assigned to all glomeruli or fields of tubules.

Picro-sirius red staining

To detect collagen accumulation in the kidney, sections were stained with picro-sirius red [15]. In brief, sections were dewaxed and incubated with 0.1% picro-sirius red in aqueous picric acid for one hour. Slides were washed in tap water and dried, then mounted. Using this method, collagens stained as red color. Collagen staining in the kidney sections were graded according to the densities of the staining: 1, mild staining; 2, intermediate staining; and 3, strong staining. This was performed under a light microscope in a masked fashion at a magnification of ×400 using the Imaging Analysis System.

Immunohistochemistry

Protein expression of fibronectin was assessed by using an immunohistochemical approach [16]. In brief,

Table 1. Sequences of forward and reverse primers and probes of collagen I and fibronectin

	Collagen I	Fibronectin
Forward primer	5′TGCCGATGTCGCTATCCA-3′	5′-CATGGCTTTAGGCGAACCA-3′
Reverse primer	5′TCTTGCAGTGATAGGTGATGTTCTG-3′	5′CATCTACATTCGGCAGGTATGG-3′
Probe	5′-CCTTCCTGCGCCTGA-3′	5′-CCCCGTCAGGCTTA-3′

sections were dewaxed, and then endogenous peroxidase was inactivated using 3% hydrogen peroxide (H_2O_2) in methanol for 20 minutes. Sections were blocked with protein blocking agent for 20 minutes. The kidney sections were incubated with a rabbit polyclonal fibronectin antibody (Dako, Carpinteria, CA, USA). Biotinylated horse antirabbit immunoglobulin (Vector Laboratories, Burlingame, CA, USA) was used as a second antibody, followed by horseradish peroxidase–conjugated streptavidin. Peroxidase activity was identified by reaction with 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma Chemical Co., St. Louis, MO, USA).

Reverse transcription-polymerase chain reaction

Three micrograms of total RNA extracted from the frozen cortex of the kidney was used to synthesize cDNA with the Superscript First Strand synthesis system for reverse transcription-polymerase chain reaction (RT-PCR) (Life Technologies BRL, Grand Island, NY, USA) [17]. Gene expressions of collagen I and fibronectin were analyzed by real-time quantitative RT-PCR performed with the TaqMan system based on real-time detection of accumulated fluorescence (ABI Prism 7700; Perkin-Elmer, PE Biosystems, Foster City, CA, USA). To control for variation in the amount of DNA available for PCR in the different samples, gene expression of the target sequence was normalized in relation to the expression of an endogenous control, 18S ribosomal RNA (rRNA) (18S rRNA TaqMan Control Reagent kit; ABI Prism 7700, PE Biosystems). Primers and TaqMan probe for collagen I and fibronectin and the endogenous reference 18S rRNA were constructed with the help of Primer Express (ABI Prism 7700). The primers and the probe specific for these genes are shown in Table 1. The amplification was performed with the following time course: 50°C for 2 minutes and 95°C for 10 minutes and 50 cycles of 94°C for 20 seconds and 60°C for 1 minute. Each sample was tested in triplicate. Results were expressed as relative to control kidneys, which were arbitrarily assigned a value of 1.

In situ hybridization

In situ hybridization for collagen I was performed using a method previously described [18, 19]. In brief, the cDNA probe coding for rat collagen I was cloned into pBluescript KS+ (Stratagene, La Jolla, CA, USA), linearized with XbaI and an antisense riboprobe was produced using T7 RNA polymerase. ^{35}S-labeled RNA probes for collagen I was prepared with transcription kits (Promega, Madison, WI, USA). Four-micron sections were incubated with ^{35}S-labeled RNA probes for collagen I at 60°C overnight in a 50% formamide humidified incubator. Slides were washed, dehydrated, air-dried, and exposed to BioMaxMR autoradiographic film for five days. The films were processed and the optical densities were quantitated by a microcomputer imaging device (MCID Imaging system, Ontario, Canada) coupled to an IBM computer. Slides were then dipped in Amersham nuclear emulsion (Ilford, Mobberley, Cheshire, UK), stored in a light-free box at room temperature for three weeks. Sections were brought to room temperature then immersed in Kodak D19 developer, washed in acetic acid, and fixed in Ilford Hypan prior to staining with hematoxylin and eosin for light microscopy.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) using Statview SE (Brainpower, Calabasas, CA, USA) on a Macintosh computer (Cupertino, CA, USA). Comparisons of group means were performed by Fisher least significant difference method. Because AER did not have a normal distribution, this parameter was analyzed after logarithmic transformation. Data are shown as mean ± SEM unless otherwise specified. A P value of less than 0.05 was viewed as statistically significant.

RESULTS

Metabolic parameters

Diabetic animals had reduced weight gain, increased HbA1c levels, and increased urine volume when compared to nondiabetic control rats (Table 2). However, these abnormal metabolic parameters were not significantly influenced by any of the drug therapies.

SBP

Mean values of serial measurements of SBP from week four to 24 after treatment are shown in Table 3. Diabetic rats had similar blood pressure over the experimental period as nondiabetic rats. Amlodipine treated rats had modestly decreased blood pressure when compared to untreated diabetic rats at weeks four, eight, and 20. Mibefradil-treated rats had similar blood pressure to untreated diabetic animals (Table 3).

Table 2. Body weight, kidney weight, blood pressure, and HbA1c (%) data

Parameters	Control	Diabetic	D + Amlodipine	D + Mibefradil
N	10	10	11	12
Body weight *g*	509 ± 15	378 ± 13[a]	350 ± 13[a]	382 ± 111[a]
Left kidney *g*	1.44 ± 0.05	2.06 ± 0.13[a]	1.66 ± 0.13[b]	1.96 ± 0.11[a]
Kidney/body weight *mg/g*	2.83 ± 0.08	5.48 ± 0.31[a]	4.51 ± 0.34[a,b]	5.32 ± 0.34[a]
Mean SBP *mmHg*	130 ± 5	136 ± 2	126 ± 3[a]	134 ± 3
HbA1c %	4.4 ± 0.5	11.1 ± 0.3[a]	10.3 ± 0.2[a]	10.3 + 0.5[a]
Urine volume *mL/24h*	16 ± 3	97 ± 15[a]	78 ± 12[a]	113 ± 8[a,c]

Mean SBP, mean systolic blood pressure of weeks 4 to 24. Left kidney weight was used to calculate the kidney/body weight ratios.
[a] $P < 0.05$ vs. control; [b] $P < 0.05$ vs. diabetic; [c] $P < 0.05$ vs. D + Amlodipine.

Table 3. Serial measurement of systolic pressure

Parameters	Control	Diabetic	D + Amlodipine	D + Mibefradil
Week 4	115 ± 4	135 ± 5[a]	112 ± 4[b]	124 ± 5
Week 8	125 ± 4	128 ± 5	115 ± 6[b]	138 ± 4
Week 12	131 ± 6	133 ± 5	135 ± 5	139 ± 6
Week 16	129 ± 5	141 ± 6	136 ± 3	142 ± 5
Week 20	132 ± 6	139 ± 6	126 ± 5[b]	128 ± 5
Week 24	131 ± 3	137 ± 4	131 ± 4	136 ± 4
Mean of 4 to 24 weeks	130 ± 5	136 ± 2	126 ± 3[b]	134 ± 3

Mean SBP, mean systolic blood pressure of weeks 4 to 24.
[a] $P < 0.05$ vs. control at same time point; [b] $P < 0.05$ vs. diabetic at same time point.

Albuminuria

AER was significantly increased in diabetic rats when compared to control rats (Fig. 1). Treatment with both amlodipine and mibefradil reduced AER in diabetic rats to a level that was still higher than that observed in control animals.

Kidney weight

Kidney weight and the ratio of kidney weight to body weight are shown in Table 2. Diabetic rats had increased kidney weight and kidney/body weight ratio when compared to control rats. Only amlodipine treatment was associated with significantly reduced kidney weight and kidney/body weight ratio compared to untreated diabetic animals.

Kidney pathology

Diabetic rats had increased glomerulosclerosis (Fig. 2A) and tubulointerstitial injury (Fig. 2B) when compared to control rats. Increased glomerulosclerosis and tubulointerstitial injury in diabetic rats were reduced by both treatments (Fig. 2).

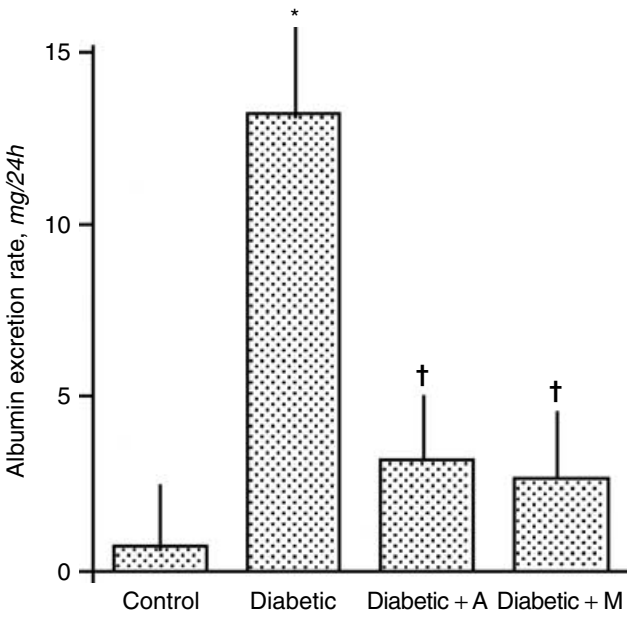

Fig. 1. Albumin excretion rate in control, diabetic, and diabetic animals treated with amlodipine (Diabetic+A) and mibefradil (Diabetic+M) are expressed as geometric mean ×/÷ tolerance factor. *$P < 0.05$ vs. control; †$P < 0.05$ vs. diabetic.

Picro-sirius red staining

Weak collagen staining was detected in the tubulointerstitium, around blood vessels, and in glomeruli in control kidneys (Fig. 3A). There was a marked increase in collagen staining in the kidneys from diabetic rats (Figs. 3B and 4). Treatment with amlodipine or mibefradil was associated with less collagen staining when compared to untreated diabetic animals (Figs. 3C, D, and 4).

Fibronectin expression

There was a three-fold increase in gene expression of fibronectin in the diabetic kidney when compared to control kidney, as assessed by RT-PCR. Elevated renal fibronectin gene expression was attenuated by both amlodipine and mibefradil therapies to a similar degree (Fig. 5). Positive fibronectin staining, as assessed by immunostaining, was detected in the glomeruli, and to a lesser extent in the tubulointerstitium. Fibronectin immunostaining was increased in the kidneys of diabetic animals (Fig. 6). Both amlodipine and mibefradil treatment attenuated fibronectin protein expression in the kidney to a similar level as that observed in control kidneys (Fig. 6).

Collagen I gene expression

There was an increase in collagen I gene expression in the diabetic kidney when compared to control kidney, as assessed by RT-PCR (Fig. 7). Attenuated collagen I gene expression was found in the kidneys of diabetic rats treated with either amlodipine or mibefradil. In situ

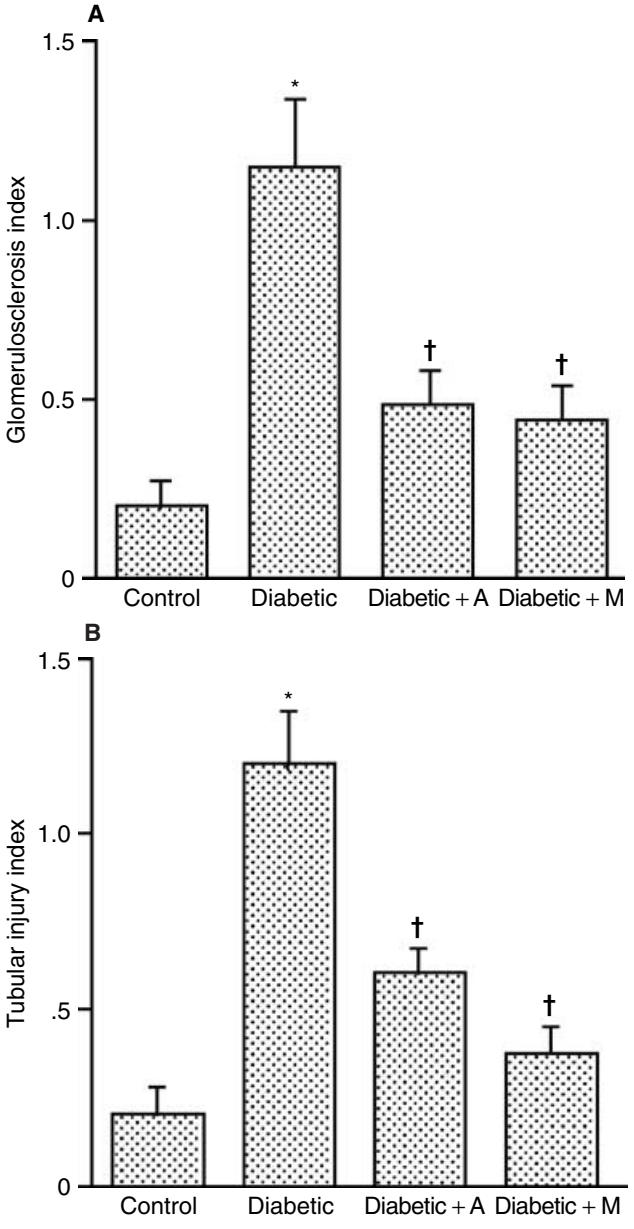

Fig. 2. Indices of glomerulosclerosis (*A*) and tubulointerstitial injury (*B*) in control, diabetic, and diabetic animals treated with amlodipine (Diabetic+A) and mibefradil (Diabetic+M) are shown as mean ± SEM. $^*P < 0.05$ vs. control; $\dagger P < 0.05$ vs. diabetic.

hybridization studies showed gene expression of collagen I predominantly in the tubulointerstitium (Fig. 8A). The increase in gene expression of collagen I was also detected in the kidneys of diabetic animals by in situ hybridization (Fig. 8). Both amlodipine and mibefradil treatments attenuated collagen I gene expression, as assessed by in situ hybridization, in the diabetic kidney (Fig. 8).

DISCUSSION

The findings of the present study have demonstrated that CCBs, including amlodipine, an L-channel CCB, and mibefradil, a T- as well as L-channel CCB, attenuate the development of albuminuria, prevent kidney structural injury, and reduce the renal production of extracellular matrix proteins in normotensive diabetic rats. These findings suggest that CCBs may be useful in preventing pathologic injury in the diabetic kidney.

Agents that interrupt the RAS, such as ACE inhibitors and ARBs, have been recommended as first line drug therapy for diabetic patients with albuminuria [20]. However, in contrast to the data obtained with RAS blockers, the effects of CCBs on diabetic patients with albuminuria are conflicting. Some clinical studies have found that CCBs are effective in reducing albuminuria [1, 21], others have reported neutral effects [22, 23], and indeed others have demonstrated that CCBs may be deleterious for the kidney [24]. A lack of consistent findings for CCBs in human diabetic nephropathy may be related to a number of factors, including patient selection (type 1 or type 2 diabetes), duration of diabetes, the degrees of underlying renal injury, the presence or absence of hypertension, and the different pharmacologic profiles of the CCBs used in the various clinical studies.

In normotensive patients with type 1 diabetes, the ACE inhibitor perindopril has been shown to be more effective than the dihydropyridine CCB nifedipine in delaying the progression of diabetic nephropathy and reducing urinary albumin excretion [6]. In a study of type 2 diabetic patients, both valsartan, an ARB, and amlodipine, a CCB, achieved a similar degree of blood pressure reduction, and yet valsartan lowered the albumin excretion rate more than amlodipine [7]. Indeed, these findings in patients with type 2 diabetes and microalbuminuria were also seen in the subgroup with baseline normotension [7]. This suggests that the antiproteinuric effect of valsartan is partly independent of blood pressure reduction [7].

It is possible that the renal protection conferred by antihypertensive agents relates not only to their action on systemic blood pressure, but also their actions on the renal microcirculation. Both approaches interrupt the RAS (ACE inhibitors and ARBs), and CCBs reduce systemic blood pressure, and this is clearly important for these agents' renoprotective properties. However, these agents have different actions on the renal microcirculation, particularly on modulating afferent and efferent arteriolar tone [25, 26]. It has been demonstrated that there is increased intraglomerular pressure in the context of diabetes due to afferent arteriolar vasodilatation even in the absence of systemic hypertension, which then promotes the development of glomerular injury, including increased albumin excretion and sclerosis [27]. It has been shown that ACE inhibitors and ARBs elicit preferential vasodilation of the efferent arteriole [25], thus ameliorating glomerular hypertension and therefore conferring renal protection. It has been demonstrated that voltage-dependent L channels predominate in the afferent

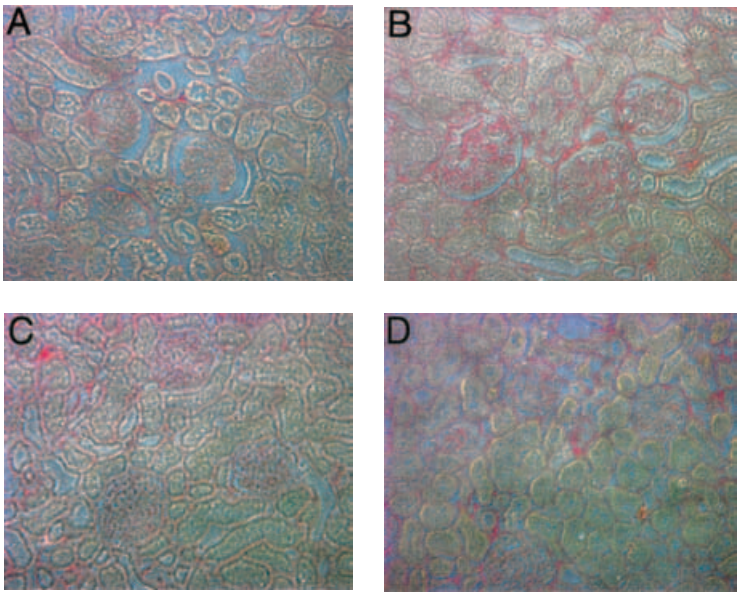

Fig. 3. Representative photomicrographs of picro-sirius red staining in control (*A*), diabetic (*B*), diabetic rats treated with amlodipine (*C*), and diabetic rats treated with mibefradil (*D*). Collagens were stained as red. Magnification ×100.

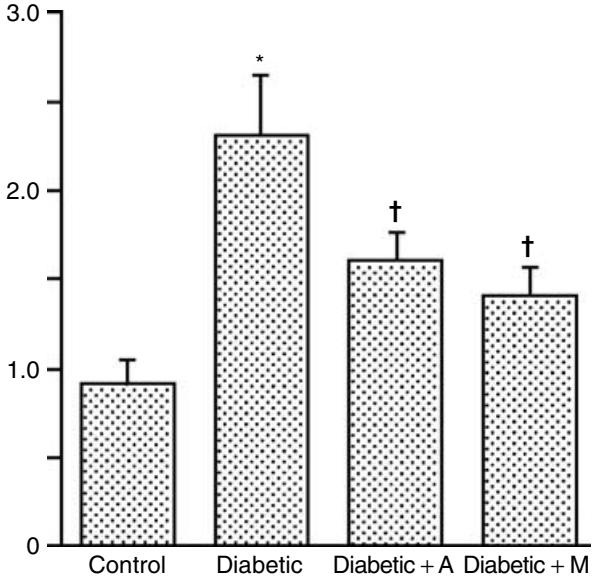

Fig. 4. Grades of picro-sirius acid staining in control, diabetic, and diabetic animals treated with amlodipine (Diabetic+A) and mibefradil (Diabetic+M) are shown as mean ± SEM. $^*P < 0.05$ vs. control; $\dagger P < 0.05$ vs. diabetic.

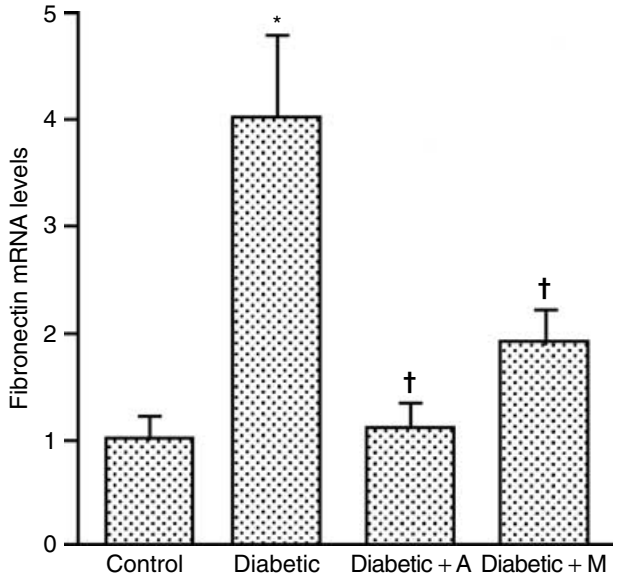

Fig. 5. Gene expression of fibronectin assessed by reverse transcription-polymerase chain reaction (RT-PCR) in control, diabetic, and diabetic animals treated with amlodipine (Diabetic+A) and mibefradil (Diabetic+M) are shown as mean ± SEM. $^*P < 0.05$ vs. control; $\dagger P < 0.05$ vs. diabetic.

arteriole but are sparse or functionally silent at the efferent arteriole [28]. Because traditional CCBs such as dihydropyridine CCBs act on L-type calcium channels, this CCB class causes preferential dilation of the afferent arteriole, with an only modest action on the efferent arteriole [26]. This may result in glomerular hypertension that could lead to progression of renal disease. This L-channel activation of certain CCBs could partly explain the unfavorable renal effects previously reported with conventional CCBs in renal disease.

However, newer CCBs, such as mibefradil, which also blocks T-type calcium channels, have been reported to dilate both afferent and efferent arterioles [29]. The T-type channel is present on juxtamedullary efferent arterioles as well as afferent arterioles of superficial and juxtamedullary nephrons [28]. Indeed, a number of studies have suggested a critical role for T-type calcium channels in modulating efferent arteriolar tone [27]. Mibefradil decreases both afferent and efferent arteriolar resistance in spontaneously hypertensive rats (SHR) [30].

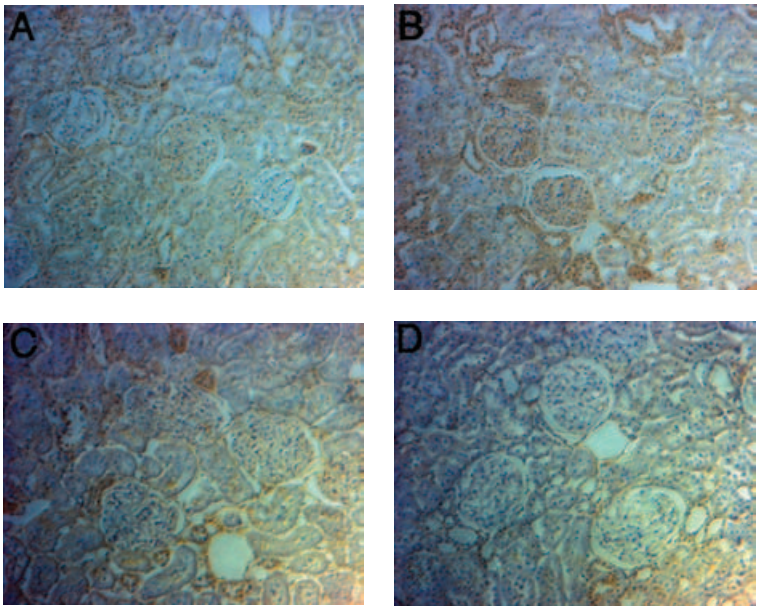

Fig. 6. Representative photomicrographs of fibronectin staining in control (*A*), diabetic (*B*), diabetic rats treated with amlodipine (*C*) and diabetic rats treated with mibefradil (*D*). Positive fibronectin was stained as brown. Magnification ×100.

Furthermore, this T-type CCB has been shown to reverse angiotensin II–induced constriction of the efferent arteriole in the isolated perfused rat hydronephrotic kidney model [27]. Based on these recent in vivo and in vitro studies, the various actions of CCBs on renal protection might be related to differences in the distribution of voltage-dependent calcium channels within the renal microvasculature and the pharmacologic properties of various CCBs.

In the present study, both amlodipine, an L-type channel CCB, and mibefradil, a T- as well as L-type CCB, attenuated increased albumin excretion in this normotensive model of diabetic nephropathy. However, amlodipine modestly reduced systolic pressure, whereas mibefradil had no significant influence on systolic blood pressure. Indeed, it is possible that the beneficial effects of amlodipine may be primarily related to its action as a blood pressure–lowering agent, whereas mibefradil conferred renoprotection primarily via its effects on glomerular arteriolar resistance.

The difference reported for CCBs in experimental renal disease may relate to the different animal models that have been examined. For example, amlodipine and mibefradil have been reported to reduce blood pressure in subtotally nephrectomized rats, yet failed to attenuate the increased proteinuria and glomerulosclerosis seen in that model [31]. Furthermore, in a model combining diabetes and hypertension, CCBs failed to attenuate albuminuria [32]. This lack of effect of CCBs may be due to the impairment of renal blood flow autoregulation by both mibefradil and amlodipine, a phenomenon which could be deleterious in the setting of systemic hypertension because it would allow direct transmission of the elevated systemic blood pressure to the glomerular microcirculation [31].

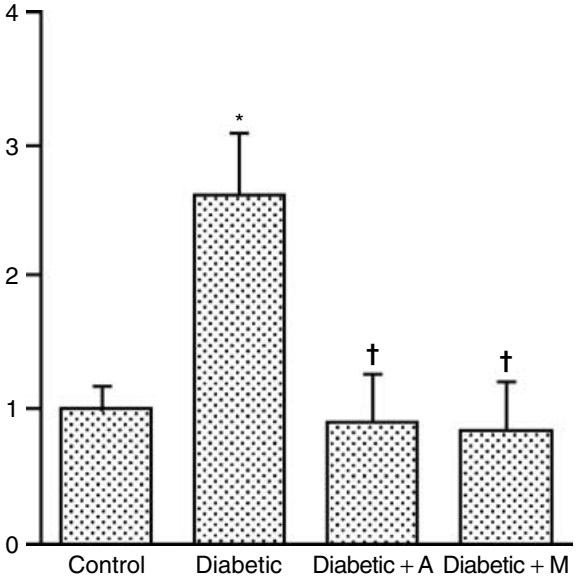

Fig. 7. Gene expression of collagen I assessed by reverse transcription-polymerase chain reaction (RT-PCR) in control, diabetic, and diabetic animals treated with amlodipine (Diabetic+A) and mibefradil (Diabetic+M) are shown as mean ± SEM. $^{*}P < 0.05$ vs. control; †$P <$ 0.05 vs. diabetic.

Increased extracellular matrix proteins in the glomeruli and tubulointerstitium have been considered major hallmarks of pathologic injury in diabetic nephropathy [3]. In a previous study, we found increased fibronectin expression in the diabetic kidney [9]. In the present study, we confirmed this finding and showed that both amlodipine and mibefradil reduced the gene and protein expression of this extracellular matrix protein in the kidneys from diabetic animals. Collagen I is also considered an important extracellular matrix protein, and in the present study,

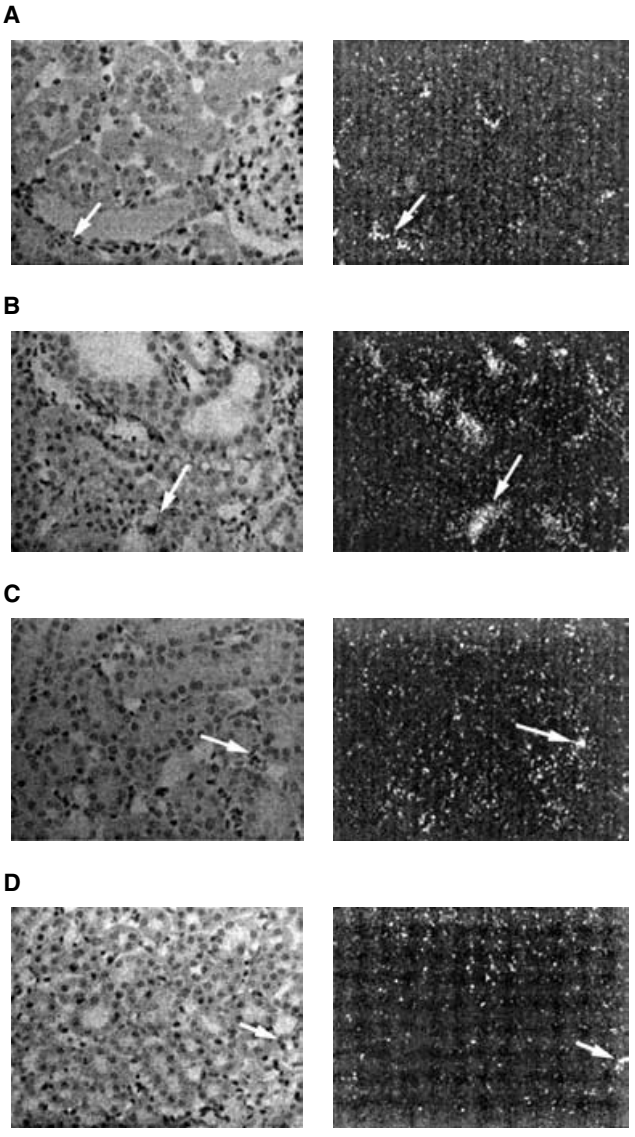

Fig. 8. Representative photomicrographs of collagen I gene expression by in situ hybridization (arrows) in control (*A*), diabetic (*B*), diabetic rats treated with amlodipine (*C*), and diabetic animals treated with mibefradil (*D*). Left panels are black and white, and right panels are dark field photomicographs.

increased gene expression of this protein was observed in the diabetic kidney using both RT-PCR and in situ hybridization techniques. Furthermore, both amlodipine and mibefradil attenuated collagen I expression. These findings suggest that CCBs influence the accumulation of multiple extracellular matrix proteins by reducing the synthesis of these proteins and that this leads to the prevention of glomerular and tubulointerstitial injury.

CONCLUSION

Both experimental and clinical studies have demonstrated that prevention of the rise in blood pressure with evolving diabetic renal disease, regardless of whether RAS blockers such as ACE inhibitors or ARBs or CCBs are used, will delay the onset of nephropathy and albuminuria. In this regard, CCBs could be used as part of a multiple drug approach including first line agents such as an ACE inhibitor or an ARB, to optimize blood pressure control, as is currently recommended by recent guidelines emphasizing aggressive blood pressure lowering in subjects with diabetes and/or renal disease [33]. However, once hypertension and advanced nephropathy are present, CCBs alone may be inappropriate as monotherapy without an ACEI or ARB. Indeed, CCBs are only indicated for the treatment of hypertension, and no major role has been established for this class of antihypertensive agents in normotensive diabetic patients [6, 7].

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